JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 1995, p. 248–250 Vol. 33, No. 1
0095-1137/95/$04.00+0

Simultaneous Identification of Strains of *Escherichia coli* Serotype O157:H7 and Their Shiga-Like Toxin Type by Mismatch Amplification Mutation Assay-Multiplex PCR

THOMAS A. CEBULA, WILLIAM L. PAYNE, AND PETER FENG*

Center for Food Safety and Applied Nutrition, Food and Drug Administration, Washington, D.C. 20204

Received 27 July 1994/Returned for modification 3 October 1994/Accepted 24 October 1994

Mismatch amplification mutation assay primers, specific for a unique base substitution in *uidA* of *Escherichia coli* O157:H7, was coupled with primers for the Shiga-like toxin I (SLT-I) and SLT-II genes in a multiplex PCR assay. Analysis of 108 bacteria showed that all *Escherichia coli* serotype O157:H7 strains were identified simultaneously with the SLT types encoded by these strains.

Enterohemorrhagic *Escherichia coli* (EHEC) of serotype O157:H7 causes hemorrhagic colitis, which may develop into life-threatening hemolytic-uremic syndrome (HUS). The pathogenicity of EHEC appears to be associated with the presence of a 60-MDa plasmid and the production of several enterotoxins (10, 14). These toxins are referred to as Shiga-like toxins (SLTs), because SLT-I closely resembles, in amino acid sequence, structure, and activity, the Shiga toxin of *Shigella dysenteriae* type 1 (17). Although more than 100 *E. coli* serotypes produce SLTs (10, 14), O157:H7 is the predominant serotype implicated in food-borne diseases. A large outbreak in 1993, traced to the consumption of undercooked hamburgers contaminated with O157:H7, infected over 700 persons in four states and resulted in 51 cases of HUS and four fatalities (4).

The absence of sorbitol fermentation by O157:H7 is a characteristic phenotype used to isolate this organism from clinical and food specimens. Though useful, confirmation with O157 and H7 antisera is required since other bacteria share this phenotype and because there are strains of O157:H7 that can ferment sorbitol (9). Antibodies to the O157 antigen are also used in many assays to detect O157:H7 isolates in clinical and food samples. These tests, however, provide no information on the toxin types produced by the isolates and are not specific, since the O157 antigen is present on other *E. coli* species (16) and anti-O157 sera often cross-reacts with *Citrobacter freundii*, *Escherichia hermanii*, and other bacteria (2). Analyses of food products with anti-O157 serum have recognized O157 isolates that neither produced SLT nor were of the H7 serotype (18). Similarly, antibody- or DNA-based assays for identifying SLT or bacteria carrying SLT genes (1, 13) will not discriminate O157:H7 isolates from the numerous other serotypes that also produce SLTs (10, 14). Although some SLT-producing, non-O157:H7 serotypes of *E. coli* have been isolated from symptomatic patients (15), the implication of these serotypes in food-borne infections is not as well established (3).

To overcome the limitations of these existing methods, we developed a multiplex PCR assay that simultaneously identifies isolates of O157:H7 and the types of SLT it encodes. The assay uses three sets of primers, two of which are directed to the conserved regions within the genes encoding for SLT-I and SLT-II (12). The third set of primers is directed to the *uidA* gene, which encodes for β-glucuronidase in *E. coli*. Although O157:H7 isolates do not exhibit glucuronidase activity, they carry the *uidA* gene (8). Earlier sequencing revealed that the *uidA* of O157:H7 had a G residue (rather than the T residue found in wild-type *E. coli*) at position 92 (7). This highly conserved base change was used to identify O157:H7 isolates by allele-specific hybridization (6). Thus, although not directly involved in pathogenicity, the conserved base change in the *uidA* allele is a powerful, though coincidental, marker of O157:H7 strains. Exploiting the uniqueness of this base change, we designed the third set of primers in a mismatch amplification mutation assay (MAMA) format (5) to preferentially amplify the *uidA* allele in O157:H7 strains. PT-2, the 20-base, upstream, allele-specific primer, carries the conserved G (rather than T) at the 3′ end and also a G (rather than A) at the 19th position. Thus, relative to the wild-type *E. coli uidA* gene, PT-2 contains two mismatched bases but only a single mismatch with respect to the *uidA* allele of O157:H7 strains (Fig. 1). The double mismatch was designed to ensure that PT-2 will not prime with the wild-type *uidA* gene of *E. coli*. The primers and the expected sizes of amplification products are shown in Table 1.

The multiplex PCR assay was performed as follows. Each 100 μl of reaction mixture contained 200 μM (each) deoxyribonucleotide, 50 pmol of each primer (Table 1), and 1× PCR buffer (50 mM KCl, 2.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 8.3). The crude cell lysate used for template DNA was prepared by heating 0.5 ml of an overnight bacterial culture for 5 min

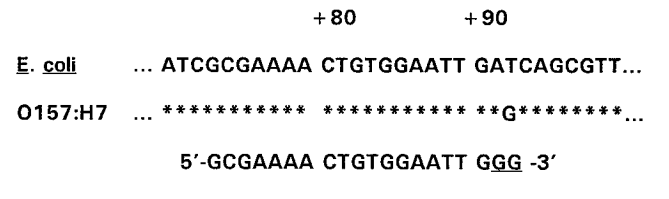

FIG. 1. Partial sequence of the *uidA* gene of *E. coli* and O157:H7, showing the position of the PT-2 primer. The consensus bases are shown with asterisks, and the mismatched bases in the primer are underlined.

* Corresponding author. Mailing address: HFS-516, CFSAN, FDA, 200 C St. SW, Washington, DC 20204. Phone: (202) 205-4518. Fax: (202) 401-7740.

TABLE 1. Primers used in MAMA-multiplex PCR to amplify specific fragments from the genes for SLT-I and SLT-II and the *uidA* gene

Target	Expected size (bp)	Primer	Sequence
SLT-I	348	LP30	5′ - CAGTTAATGTGGTGGCGAAGG - 3′
		LP31	5′ - CACCAGACAATGTAACCGCTG - 3′
SLT-II	584	LP43	5′ - ATCCTATTCCCGGGAGTTTACG - 3′
		LP44	5′ - GCGTCATCGTATACACAGGAGC - 3′
uidA	252	PT-2	5′ - GCGAAAACTGTGGAATTGGG - 3′
		PT-3	5′ - TGATGCTCCATAACTTCCTG - 3′

in a boiling water bath, freezing in ethanol-dry ice for 5 min, and centrifuging to remove debris. To prevent nonspecific amplification from SLT-I primers with *Salmonella* species and from *uidA* primers with *E. coli*, the hot-start technique was used: 85 μl of the reaction mixture was heated to 94°C for 5 min before template DNA (5 μl) and AmpliTaq DNA polymerase (2.5 U in a 10-μl reaction mixture) were added. The samples were amplified for 35 cycles, with each cycle consisting of 1.5 min at 94°C for denaturation, 1.5 min at 64°C for primer annealing, and 1.5 min at 72°C for strand elongation. Amplified products were examined by agarose gel electrophoresis in Tris-borate buffer.

We evaluated MAMA-multiplex PCR using *E. coli*, other SLT-producing *E. coli* of non-O157:H7 serotype, various genera of enteric and nonenteric bacteria, reference O157:H7 strains from the American Type Culture Collection (ATCC), and O157:H7 and O157:H$^-$ serotypes isolated from symptomatic patients, clinical specimens, and foods implicated in recent outbreaks. Analysis of amplification products by agarose gel electrophoresis showed that all reference strains of O157:H7 serotype were correctly identified simultaneously with the SLT type known to be produced by these strains (Fig. 2, lanes A to E). The clinical and food isolates of O157:H7 serotype were similarly identified by the assay (Fig. 2, lanes J to K). The amplicons generated in these experiments approximated the sizes predicted on the basis of the selected primer sets (Table 1). As anticipated, no products were amplified from wild-type *E. coli* (Fig. 2, lane F), whereas the expected toxin gene-specific products, but not O157:H7-specific products, were amplified from the two SLT-producing, non-O157:H7 serotypes examined (lanes G and H).

The results of MAMA-multiplex PCR analysis of 108 bacteria are summarized in Table 2. All isolates of O157:H7 serotype, including two O157:H$^-$ strains, were correctly identified along with the SLT types produced by each isolate. The two O157:H$^-$ strains were isolated from HUS patients and found to be nonmotile and to produce SLT-II as determined by Vero cell cytotoxicity assays (11). Allele-specific hybridization showed both these strains to carry the conserved base change in *uidA* (6). Several SLT-producing, non-O157:H7 serotypes of *E. coli* examined produced only toxin gene-specific amplification products. The type of SLT identified by the multiplex assay correlated well with the Vero cell cytotoxicity data (when available) for these isolates (3). Among non-*E. coli* strains tested, only *S. dysenteriae* showed an SLT-I-like amplicon. The Shiga toxin of *S. dysenteriae* type 1 is almost identical to the SLT-I of O157:H7 (17); therefore, this result was not unexpected. Although the MAMA-multiplex assay will not discriminate between *S. dysenteriae* type 1 and non-O157 EHEC serotypes that produce only SLT-I, the *uidA*-specific primers readily distinguished *S. dysenteria* type 1 species from O157:H7 isolates.

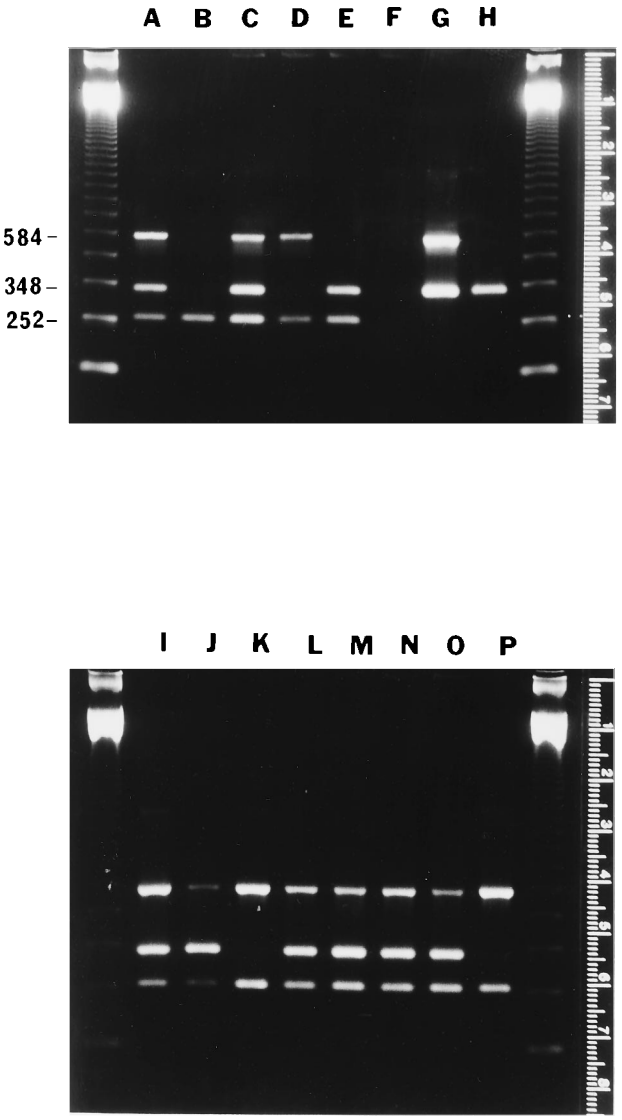

FIG. 2. Agarose gel electrophoresis of DNA fragments amplified by MAMA-multiplex PCR from selected *E. coli* strains. Lanes A to E are ATCC reference strains of O157:H7 serotype, and the SLT types known to be produced are shown below in parentheses. Lanes: A and I, 43895 (SLT-I and SLT-II); B, 43888 (none); C, 35150 (SLT-I and SLT-II); D, 43889 (SLT-II); E, 43890 (SLT-I); F, *E. coli* FDA EC227 (none); G, FDA403, O111:NM (SLT-I and SLT-II); and H, FDA400, O26:H11 (SLT-I). Lanes J to P are strains of O157:H7 serotype isolated from human and clinical samples and various foods implicated in outbreaks. Flanking lanes show 123-bp molecular size ladders; sizes marked at the left are in base pairs.

This study shows that the MAMA-multiplex PCR assay is a highly effective means for specifically characterizing EHEC organisms isolated from clinical and food specimens. The major advantage of this method over existing assays is that it can identify the types of SLT encoded by the strain and at the same time discriminate other SLT-producing *E. coli* from O157:H7, the predominant serotype implicated in disease. We are exploring the potential of MAMA-PCR as a rapid diagnostic tool for screening O157:H7 isolates directly in clinical specimens and food samples.

TABLE 2. MAMA-multiplex PCR analysis of bacteria with primers specific for genes of SLT-I and SLT-II and for the *uidA* gene

Bacterial species	Serotype	No. of isolates tested	MAMA-multiplex PCR finding			
			Product			No. of isolates in which indicated product was observed
			SLT-I	SLT-II	*uidA*	
E. coli	Wild type	8	−	−	−	8
	O157:H7[a]	42	+	+	+	33
			−	+	+	7
			+	−	+	1
			−	−	+	1
	O157:H^{-}[b]	2	−	+	+	2
	O111:NM	1	+	+	−	1
	O26:H11/H^{-}	3	+	−	−	3
	O153:H2	1	+	−	−	1
	O68:H^{-}	1	+	+	−	1
	O^{-}:H11	1	+	−	−	1
	Other[c]	11	−	−	−	11
Shigella spp.		5	−	−	−	5
S. dysenteriae	Type 1	3	+	−	−	3
Salmonella spp.		18	−	−	−	18
Other genera[d]		12	−	−	−	12

[a] Included are ATCC reference strains and strains isolated from patients (donated by P. Tarr, Children's Hospital and Medical Center, Seattle, Wash.) and from hamburger and raw milk samples implicated in outbreaks (donated by the Departments of Health of Washington and Oregon).
[b] Obtained from H. Karch, University of Würzburg).
[c] Included are serotypes O143, O78:H11, O55:NM, O127, O25:K98, O78:H11, O78:K80:H12, O25:K98:NM;O111, and O124:NM.
[d] *Enterobacter cloacae*, *Proteus vulgaris*, *Klebsiella pneumoniae*, *Serratia marcescens*, *Vibrio cholerae*, *Pseudomonas aeruginosa*, *Aeromonas hydrophila*, *Campylobacter* spp., *Staphylococcus* spp., and *Streptococcus pyogenes*.

We thank P. I. Tarr, Children's Hospital and Medical Center, Seattle, Wash.; J. Lewis, Washington State Department of Health; and H. Karch, University of Würzburg, Würzburg, Germany, for providing isolates. We thank L. Tomlinson, FDA, Washington, D.C., for editorial assistance.

REFERENCES

1. **Barrett, T. J., J. H. Green, P. M. Griffin, A. T. Pavia, S. M. Ostroff, and I. K. Wachsmuth.** 1991. Enzyme-linked immunosorbent assay for detecting antibodies to Shiga-like toxin I, Shiga-like toxin II, and *Escherichia coli* O157:H7 lipopolysaccharide in human serum. Curr. Microbiol. **23:**189–195.
2. **Bettelheim, K. A., H. Evangelidis, J. L. Pearce, E. Sowers, and N. A. Strockbine.** 1993. Isolation of a *Citrobacter freundii* strain which carries the *Escherichia coli* O157 antigen. J. Clin. Microbiol. **31:**760–761.
3. **Bokete, T. N., C. M. O'Callahan, C. R. Clausen, N. M. Tang, N. Tran, S. L. Moseley, T. R. Fritsche, and P. I. Tarr.** 1993. Shiga-like toxin-producing *Escherichia coli* in Seattle children: a prospective study. Gastroenterology **105:**1724–1731.
4. **Centers for Disease Control.** 1993. Update: multistate outbreak of *Escherichia coli* O157:H7 infections from hamburgers—western United States, 1992–1993. Morbid. Mortal. Weekly Rep. **42:**258–263.
5. **Cha, R. S., H. Zarbl, P. Keohavong, and W. G. Thilly.** 1992. Mismatch amplification mutation assay (MAMA): application to the c-H-*ras* gene. PCR Methods Applic. **2:**14–20.
6. **Feng, P.** 1993. Identification of *Escherichia coli* O157:H7 by DNA probe specific for an allele of *uid*A gene. Mol. Cell. Probes **7:**151–154.
7. **Feng, P., and K. A. Lampel.** 1994. Genetic analysis of *uid*A gene expression in enterohemorrhagic *Escherichia coli* serotype O157:H7. Microbiology **140:**2101–2107.
8. **Feng, P., R. Lum, and G. W. Chang.** 1991. Identification of *uidA* gene sequences in β-D-glucuronidase-negative *Escherichia coli*. Appl. Environ. Microbiol. **57:**320–323.
9. **Fratamico, P. M., R. L. Buchanan, and P. H. Cooke.** 1993. Virulence of an *Escherichia coli* O157:H7 sorbitol-positive mutant. Appl. Environ. Microbiol. **59:**4245–4252.
10. **Griffin, P. M., and R. V. Tauxe.** 1991. The epidemiology of infections caused by *Escherichia coli* O157:H7, other enterohemorrhagic *E. coli*, and the associated hemolytic uremic syndrome. Epidemiol. Rev. **13:**60–98.
11. **Gunzer, F., H. Böhm, H. Rüssmann, M. Bitzan, S. Aleksic, and H. Karch.** 1992. Molecular detection of sorbitol-fermenting *Escherichia coli* O157 in patients with hemolytic-uremic syndrome. J. Clin. Microbiol. **30:**1807–1810.
12. **Jackson, M. P., R. J. Neill, A. D. O'Brien, R. K. Holmes, and J. W. Newland.** 1987. Nucleotide sequence analysis and comparison of the structural genes for Shiga-like toxin I and Shiga-like toxin II encoded by bacteriophages from *Escherichia coli* 933. FEMS Microbiol. Lett. **44:**109–114.
13. **Karch, H., and T. Meyer.** 1989. Single primer pair for amplifying segments of distinct Shiga-like-toxin genes by polymerase chain reaction. J. Clin. Microbiol. **27:**2751–2757.
14. **Karmali, M. A.** 1989. Infection by verocytotoxin-producing *Escherichia coli*. Clin. Microbiol. Rev. **2:**15–38.
15. **Mariani-Kurkdjian, P., E. Denamur, A. Milon, B. Picard, H. Cave, N. Lambert-Zechovsky, C. Loirat, P. Goullet, P. J. Sansonetti, and J. Elion.** 1993. Identification of a clone of *Escherichia coli* O103:H2 as a potential agent of hemolytic-uremic syndrome in France. J. Clin. Microbiol. **31:**296–301.
16. **Rice, E. W., E. G. Sowers, C. H. Johnson, M. E. Dunnigan, N. A. Strockbine, and S. C. Edberg.** 1992. Serological cross-reactions between *Escherichia coli* O157 and other species of the genus *Escherichia*. J. Clin. Microbiol. **30:** 1315–1316.
17. **Strockbine, N. A., M. P. Jackson, L. M. Sung, R. K. Holmes, and A. D. O'Brien.** 1988. Cloning and sequencing of the genes for Shiga toxin from *Shigella dysenteriae* type 1. J. Bacteriol. **170:**1116–1122.
18. **Willshaw, G. A., H. R. Smith, D. Roberts, J. Thirlwell, T. Cheasty, and B. Rowe.** 1993. Examination of raw beef products for the presence of Vero cytotoxin producing *Escherichia coli*, particularly those of serogroup O157. J. Appl. Bacteriol. **75:**420–426.